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Comparison of PCR, BIO-PCR, DIA, ELISA and isolation on semiselective medium for detection of *Xanthomonas albilineans*, the causal agent of leaf scald of sugarcane

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Polymerase chain reaction (PCR) and newly designed primers, XAF1/XAR1, were tested for selective detection of the causal agent of leaf scald of sugarcane, *Xanthomonas albilineans*. The efficiency and reliability of PCR were compared with dot immunobinding assay (DIA), ELISA and classical isolation techniques for detecting *X. albilineans* in suspensions of pure cells and extracts of field-collected stalk and leaf samples of sugarcane. In addition, classical PCR and BIO-PCR (biological amplification followed by PCR) were compared with isolation on a semiselective agar medium. Classical PCR and BIO-PCR techniques had the advantage of not requiring pathogenicity tests to confirm the identity of colonies tentatively identified as *X. albilineans* on modified semiselective XAM agar medium. The m-XAM medium and BIO-PCR techniques were the most sensitive; however, the former required seven days whereas the latter required only four days. The BIO-PCR technique was as sensitive as the semiselective medium technique and eliminated the need to conduct any additional tests to confirm the identification.

Keywords: bio-PCR, leaf scald, PCR detection, sugarcane, *Xanthomonas albilineans*

Introduction

Leaf scald (LS), caused by *Xanthomonas albilineans*, is one of the major diseases of sugarcane (interspecific hybrids of *Saccharum* spp.) and occurs in more than 60 geographical locations throughout the world (Ricaud & Ryan, 1989; Rott, 1995). The disease is characterized by chronic and acute symptoms including: pencil-line leaf streaks, chlorotic leaf streaks either with or without necrosis, and reddish discoloration of vascular bundles at the nodes of the stalk (Ricaud & Ryan, 1989). Latent infection frequently occurs, making visual diagnosis impossible (Ricaud & Ryan, 1989). Consequently, the pathogen can be spread by planting infected seed cane used for propagation. This accounts for intercountry transmission of LS during the exchange of the sugarcane germplasm and the spread of LS locally in seed cane during commercial planting.

Current methods for LS detection rely on either culturing on general plating media, such as Wilbrink's (Ricaud & Ryan, 1989) or on a semiselective medium for *X. albilineans* (Davis *et al*., 1994), or serological assays (enzyme-linked immunosorbent assay [ELISA], dot immunobinding assay [DIA], and tissue blot immunosorbent assay [TBIA]) using either monoclonal or polyclonal antisera (Tsai *et al*., 1990; Comstock & Irey, 1992; Rott *et al*., 1994). These assays have minimum threshold levels of around 10^5 cfu mL^{-1} for detection and, therefore, may not always accurately detect *X. albilineans* in infected sugarcane stalks that show no symptoms (Irey & Comstock, 1991). Although culturing on semiselective medium is much more sensitive than serological methods and enables the detection of a low titre of bacteria, the pathogen requires 6–8 days to form characteristic colonies, thus, restricting the use of isolation as a means of diagnosis (Rott, 1995). Clearly, a sensitive and more rapid technique is needed both for quarantine purposes and in epidemiological research. Polymerase chain reaction (PCR) (Saiki *et al*., 1988) is one such technique; it has become a powerful detection and diagnostic technique for plant pathogenic bacteria (Prosen *et al*., 1993; Audy *et al*., 1996; Hartung *et al*., 1996; Slack *et al*., 1996). A PCR-based detection system using two tRNA gene primers is available for sensitive detection of *X. albilineans*-infected sugarcane; however, with *X. campestris* pv. *vasculorum* a faint false positive band occasionally occurred on agarose gel after electrophoresis (Honeycutt *et al*., 1995). Thus, the PCR product is not unique to the pathogen. Several other

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Table 1 List of bacterial isolates

Isolates[a]	Source[b]	Geographic origin	PCR[c]
X. albilineans			
FB500* (921108)	1	Florida	+
FB502* (921254)	1	Florida	+
FB504* (931 – A)	1	Florida	+
FB508 (931 – B)	1	Florida	+
FB514* (932d – 1)	1	Florida	+
FB575* (GP5)	2	Guadeloupe	+
FB576* (HV5)	2	Burkina Faso	+
FB578* (083 A)	2	USA	+
FB579*(KNA,03 A)	2	St. Kitts	+
FB581* (G55)	2	Guadeloupe	+
FB583* (MQE58)	2	Martinique	+
FB648* (33915)	3	Fiji	+
93001	4	Florida	+
93002	4	Florida	+
93003	4	Florida	+
93012	4	Florida	+
93013	4	Florida	+
93014	4	Florida	+
93018	4	Florida	+
X. campestris pv. *citri**			
(LX05)	5	PRC	–
X. c. pv. *aurantifolii**			
(XC64)	6	Argentina	–
X. c. pv. *citrumelo**			
(XC34)	2	Florida	–
X. c. pv. *vasculorum**			
FB571* (11408)	2	Reunion	–
FB572* (11412)	2	Mauritius	–
FB573* (11413)	2	Madagascar	–
FB574* (11414)	2	South Africa	–
X. c. pv. *campestris*			
FB 1020 (33913)	3	United Kingdom	–
X. oryzae pv. *oryzae**			
FB 215 (35933)	3	India	–
X. o. pv. *oryzicola**			
FB 222 (49072)	3	Malaysia	–
yellow saprophytic bacteria			
93–1-1	4	Florida	–
93–1-2	4	Florida	–
93–3-4	4	Florida	–
93–83	4	Florida	–
93–189	4	Florida	–
Escherichia coli			
(K-12)	7	Maryland	–
Pseudomonas syringae pv. *syringae*			
FC-1 (C-199)	7	Idaho	–

[a]Genomic DNA was extracted from isolates marked *, at USDA-ARS, Foreign Diseases–Weed Research Unit, Frederick, MD 21702.

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[c]Presence (+) or absence (–) of 600 bp target product.

PCR primers specific for *X. albilineans* have been identified in preliminary reports (Davis *et al*., 1995; Hatziloukas *et al*., 1995; Pan *et al*., 1995). Recently, a PCR protocol for the detection of *X. albilineans* was described (Pan *et al*., 1997).

A comparison between classical PCR, BIO-PCR, ELISA, DIA and isolation on semiselective medium for detection of *X. albilineans* in cell suspensions from pure culture and in vascular bundle sap extracted from naturally infected sugarcane stalks and leaves is reported here.

Materials and methods

Bacterial isolates and culture conditions

Bacteria used to test PCR primer specificity are listed in Table 1. All cultures were stored in 30% glycerol at − 80°C. The semiselective agar medium XAM (Davis *et al*., 1994) was modified by adding 5 g yeast extract per litre (m-XAM). Isolation of *X. albilineans* from vascular bundle sap of stalks, and leaves of sugarcane, weeds and soil samples, was conducted on m-XAM. Cultures of *X. albilineans* were grown on m-XAM agar plates for 7 days at 28°C, and the cells suspended in sterile distilled water, adjusted to 0·1 OD at 600 nm and diluted for use in cell recovery experiments.

Sugarcane field samples

Four different field samples of sugarcane were collected, by cutting sections of stalk near the soil line (one stalk per plant), to compare detection techniques. The first sampling (15 cm sections) was taken from 155 four-month-old plants. These had been inoculated at planting, five months earlier, with *X. albilineans* isolate 93001, by spraying the cut ends of single-bud seedpieces with a suspension containing 10^8 cells mL^{-1} using a high pressure (280 kPa) atomizing paint spray nozzle. The presence or absence of symptoms on each individual plant was recorded at sampling. The second sampling totalled 200 stalks (50-cm sections) of 12-month-old plants growing in a cultivar development plot at the Canal Point Sugarcane Field Station, where the disease was known to be present. Plants were selected from 20 different clones, and each collection contained five stalks with symptoms and five without. The third sampling included 225 mature stalks of the moderately susceptible cultivar CP 80–1743, ranging in age from 10 to 12 months, taken at random from five commercial sugarcane fields (45 sections of stalk per field, each section 50 cm). The disease status of these stalks was unknown since samples were taken during harvest, just after a preharvest burn. The fourth field sampling consisted of 150 stalks of CP 80–1743 growing in a commercial sugarcane field where the disease was present. These were taken at random during harvest after the field was burned and were used to compare *X. albilineans* detection by isolation on m-XAM, PCR and BIO-PCR.

Extraction of sap from vascular bundles of sugarcane stalks

Sap was extracted from symptom-bearing stalks cut from the base of mature plants (Comstock & Irey, 1992). Five cores (1·5 cm long by 0·8 cm diameter) were removed using a sterilized 8 mm cork borer. Each core was centrifuged in a 1·5 mL microcentrifuge tube at 8160 ***g*** for 5 min. This resulted in ≈ 100 μl of sap per core from the vascular bundles. The core and supernatant were then discarded and the pellets were resuspended in a volume of sterile distilled water equal to the supernatant. Suspended pellets from the same stalk were combined and distributed into 100 μl aliquots in 1·5 mL microcentrifuge tubes. After centrifuging, the supernatant was discarded and different reagents were used to resuspend the pellets, depending on the assays. For ELISA, the pellets were resuspended in 100 μl of 50 mM carbonate–bicarbonate coating buffer (CB), pH 9·6. For DIA they were resuspended in 100 μl of 0·02 M Tris-HCl, 0·15 M NaCl, pH 7·5 (TBS) and for m-XAM in 100 μl sterile deionized water. For classical PCR assays the pellets were washed once in filter-sterilized deionized water (FSDW), resuspended in 100 μl FSDW, and boiled for 10 min in a water bath. Samples for PCR assays were stored at either – 20°C or 4°C. Stalks without symptoms were sampled using the same procedure, except that the pellets were resuspended, pooled, centrifuged and resuspended in a quarter of the original volume, to enrich the amount of target. A cell suspension from a pure culture of *X. albilineans* (93001) containing ≈ 10^8 cfu mL^{-1}, and sap extracted from healthy stalks, were used as positive and negative controls, respectively.

Preparation of leachates from sugarcane leaves

Leaves of experimental clones with or without typical pencil-line leaf scald symptoms were collected separately from noninoculated field plants at the Canal Point Sugarcane Field Station. Working in different laboratories of the building to avoid cross-contamination, a 1·5 × 14 cm section of leaf tissue (≈ 500 mg) was cut from several leaves with and without symptoms, and soaked in 1·5 mL deionized water plus 0·1% polyvinyl-pyrrolidone, MW 40 000 (PVP-40), in sterile 50 mL centrifuge tubes, for 24 h at 25°C to allow bacteria to diffuse from the exposed vascular tissue. The entire leachate was transferred to a microcentrifuge tube and centrifuged at 8160 **g** for 5 min. The resulting pellet was resuspended in 100 μL FSDW. After boiling for 10 min or treating with 50 mM NaOH to remove possible inhibitors, all samples were stored at 4°C for later testing (within one month).

Polymerase chain reaction assay

Primers

Primers were designed based on sequences of an anonymous PCR fragment amplified and cloned from genomic DNA of a *X. albilineans* type A isolate (33915, ATCC). One clone, pEHRCSA-4b, was sequenced. Based on the sequence information, a primer pair, XAF1/XAR1, was designed, that was specific for *X. albilineans* by Southern hybridization (Hatziloukas *et al*., 1995). The primer sequences were: XAF1: 5′-CCT GGT GAT GAC GCT GGG TT-3′ and XAR1: 5′-CGA TCA GCG ATG CAC GCA GT-3′. Either linearized DNA of plasmid pEHRCSA-4b or the PCR product amplified from the genomic DNA of *X. albilineans* (33915) was labelled using a nonradioactive DNA labelling kit (Boehringer Mannheim Biochemicals, Indianapolis, IN) and probed against target DNA immobilized on Nytran membranes (Scheicher and Schuell, Keene, NH). The blots were washed twice in 2 × SSC–0·1% SDS (SSC: saline sodium citrate, 0·3 M NaCl, 0·03 M Na-citrate; SDS: sodium dodecyl sulfate) at 65°C and twice in 0·1 × SSC–0·1% SDS at 65°C. XAF1/XAR1 primers were synthesized by Life Technologies (Grand Island, NY, Gibco BRL, Lot no. 545726).

Amplification conditions

The protocol was as follows: 1 μl template and 3 μl of 120 μM XAF1/XAR1 primer mixture was overlaid with one drop of mineral oil and denatured at 100°C for 7 min. The mixture was then held at 80°C while the remaining 25 μl reaction mixture was added (0·25 μl of 100 μM deoxynucleotide mix [dNTPs][Sigma, St. Louis, MO], 0·125 μl of 0·625 U AmpliTaq DNA polymerase [Perkin Elmer Cetus, Foster City, CA], 2·5 μl of 10 × PCR buffer II pH 8·3, 1·5 μl of 1·5 mM $MgCl_2$, and 16·6 μl FSDW). Ten cycles were used at 94°C for 45 s for denaturing; 65°C for 1 min for annealing; and 72°C for 1 min for extension. This was followed by 10 cycles identical to the previous 10 except for a 2 min extension, and another 10 cycles with a 3 min extension. Final extension was at 72°C for 10 min. These conditions were used for all comparative experiments. PCR amplification products were detected by electrophoresis through a 1·5% agarose gel, stained with 0·5 μg mL^{-1} ethidium bromide, visualized on a 302 nm ultraviolet transluminator, and photographed using Polaroid film 667 or printed image through IS-1000 Digital Image System (Alpha Image Inc., San Leandro, CA).

BIO-PCR assays were performed as described (Schaad *et al*., 1995). Cores of sugarcane stalks were centrifuged as above, the pellets resuspended in equal volumes of FSDW, and 35 μl aliquots plated onto each of three plates of m-XAM. Plates were incubated for four days at 28°C to allow viable populations of *X. albilineans* to grow into pin-point-sized colonies. Tests showed that suspensions containing very few cells (100 or less per ml) required 4 days to produce pin-point-sized colonies. Cells were washed from the plate three times using 1 mL aliquot of FSDW. The 3 mL pooled sample was

processed, as described for classical PCR, except that 35 μl of cell suspension was used in 50 μl total volume per reaction.

Genomic DNA preparation

For extraction of genomic DNA, a modified cetyltrimethylammonium bromide (CTAB) method (Murray & Thompson, 1980) was used with $\approx$ 1 g (wet weight) of bacteria grown for 72 h in Luria-Bertani (LB) liquid shake culture at 25°C. All extractions were conducted under partial containment at the Foreign Disease–Weed Science Research Laboratory, Frederick, MD. Bacterial cultures were centrifuged (3000 ***g*** for 30 min) and pellets washed twice in TES buffer (10 mM Tris, 50 mM EDTA, 100 mM NaCl, pH 8·0). Pellets were resuspended in 20 mL TES buffer containing proteinase K (0·625 mg g^{-1}) and 1 mL of 20% SDS (0·25 g g^{-1}), and incubated at 55°C for 4–5 h. Extracellular polysacchrides were removed by adding 5 M NaCl containing 10% CTAB and incubated at 65°C for 30 min. Protein was removed by adding an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1 v/v/v) and mixing gently by inversion for 5 min followed by centrifuging at 3000 ***g*** for 30 min. The aqueous phase was removed and the phenol:chloroform:isoamyl alcohol extraction was repeated. After adding 2 volumes of ice-cold 95% ethanol and 1/10 volume 3 M sodium acetate (pH 5·2), the DNA was spooled out and washed in prechilled 70% ethanol. To the DNA, 1 mL TE buffer (1 mM Tris-HCl, 1 mM EDTA) and 25 μl (10 mg mL^{-1}) RNAase were added and incubated at 37°C for 3 h. DNA was isolated by phenol/chloroform extraction, as described above, and the DNA dissolved in TE buffer. The DNA concentration and the purity (260/280 ratio) was determined spectrophotometrically at A_{260nm} and A_{280nm}. DNA stock (1 μg mL^{-1}) and working (25 ng mL^{-1}) solutions were prepared for assays.

DNA samples were also prepared from pure cultures of *X. albilineans,* and several unknown yellow-pigmented bacterial saprophytes found on isolation plates of sugarcane vascular bundle sap (Table 1), soil, and weed samples collected adjacent to sugarcane plants. Several other xanthomonads were tested, including *X. campestris* pv. *campestris* (33913), *X. c.* pv. *vasculorum* (FB571, FB572), *X. campestris* pv. *citri* (LX05), *X. oryzae* pv. *oryzae* (35933) and X. *o.* pv. *oryzicola* (49072). Bacterial cultures for PCR assays were diluted in FSDW and boiled in a water bath for 10 min.

Sensitivity of detection

The sensitivity of PCR detection of *X. albilineans* using optimal amplification conditions was compared with isolation on m-XAM, DIA and ELISA techniques. Suspensions of *X. albilineans* from pure cultures (adjusted to $A_{600nm} = 0{\cdot}1$) were diluted 10-fold serially (10^{-1}–10^{-8}) in either sterile water or filter sterilized vascular bundle sap extracted from stalks. One μl aliquots were removed from the serial dilutions for DIA, ELISA and PCR assays. To determine the colony-forming units (cfu) for the above dilutions, 1 μl of each dilution was mixed with 25 μl sterilized water, and the entire amount plated onto each of three m-XAM plates. The plates were incubated for 7 days at 28°C. The cfu numbers used in assays were expressed by multiplying by 1000 to determine viable cfu mL^{-1} in the assay reaction solution. Either sterile water or filter sterilized sap was used as a negative control for each experiment. The sensitivity of PCR detection was determined using 10-fold (100 ng to 1 pg) serial dilutions of a 1 μg mL^{-1} stock DNA solution of *X. albilineans* (33915). Experiments were repeated at least three times.

Comparison of classical PCR and BIO-PCR techniques

The techniques were compared in a test similar to that previously described for determining sensitivity. Cells of *X. albilineans* from six-day-old agar plate cultures were suspended in sterile water adjusted to an absorbance of 0·1 (A_{600nm}) and a 10-fold serial dilution series was performed. Besides detecting *X. albilineans* alone in pure culture, it was mixed with a yellow saprophyte commonly isolated from sugarcane stalks to determine if that organism might interfere with detection. Suspensions of *X. albilineans* and the yellow saprophyte were prepared by mixing an equal concentration of each organism (saprophyte from two-day-old cultures) and dilutions (10-fold), as above. For BIO-PCR, a 35 μl cell suspension of each stepwise dilution was plated onto m-XAM for biological amplification. After 4 days at 28°C, the culture plates were washed three times with 1 mL FSDW and 35 μl of the pooled sample used for PCR. For classical PCR, 35 μl of each stepwise dilution was used. A total reaction volume of 50 μl was used in both assays. Classical PCR and BIO-PCR assays were conducted on extracts of 150 sugarcane stalks collected at random from a commercial field where LS was known to be present.

Dot immunobinding assay

DIA was conducted using nitrocellulose membranes (Bio-Rad, Hercules, CA) according to the protocol of Rott (Rott *et al*., 1994). A 1:10 000 dilution of XACP-5 antiserum to *X. albilineans* (M. J. Davis, University of Florida, Homestead) was used as the primary antibody, followed by a 1:4000 dilution of goat antirabbit IgG conjugated with alkaline phosphatase (Sigma Chemical Co., St. Louis, MO) as the secondary antibody. One hundred μl aliquots of either cell suspension or sugarcane sap were loaded on 8 × 11 cm nitrocellulose membrane using a vacuum filtration apparatus (Manifold I, Schleicher & Schuell, Inc., Keene, NH). After filtering, the membrane was removed and baked at 80°C for 30 min. It was washed once in TBS-Triton X-100 (TBST) for 5 min with gentle agitation, then in the TBST

Table 2 Comparison of isolation, DIA, ELISA and PCR techniques in detecting *Xanthomonas albilineans* cells from pure culture and vascular bundle sap extracted from sugarcane stalks

Culture suspensions[a]				Vascular bundle sap[b]			
Isolation ($\times 10^3$)[c]	ELISA[d]	DIA[e]	PCR[f]	Isolation ($\times 10^3$)[c]	ELISA[d]	DIA[e]	PCR[f]
26 000	0·126 (+)	+	+	20 000	0·135 (+)	+	+
2660	0·110 (+)	+	+	2067	0·115 (+)	+	+
510	0·076 (+)	–	+	347	0·056 (+)	–	+
93	0·025 (–)	–	+	110	0·030 (–)	–	+
20	0·012 (–)	–	+	9	0·015 (–)	–	+
6	0·011 (–)	–	–	1	0·010 (–)	–	–
1	0·008 (–)	–	–	0	0·000 (–)	–	–
0	0·006 (–)	–	–	0	0·005 (–)	–	–

[a]Cells of *X. albilineans* from six-day-old cultures were suspended in sterile water ($A_{600nm} = 0{\cdot}1$) and a 10-fold dilution series (10^{-1}–10^{-8}) was performed. One μL of each stepwise dilution was used for each assay, in 25 μL total volume.

[b]Vascular bundle sap was filter-sterilized and spiked with cells of *X. albilineans*. Dilution series were then conducted and assayed as described above.

[c]Numbers of colony forming units (cfu) of *X. albilineans* in each ml of pure culture or sugarcane extract per assay were determined in triplicate using 1 μl aliquots of dilution mixed in 25 μl sterile water, spread on m-XAM semiselective medium and incubated for 7 days at 28°C.

[d]ELISA readings of 0·05 A_{405nm} or higher were considered positive.

[e]Presence (+) or absence (–) of dark blue precipitate on nitrocellulose membrane.

[f]Presence (+) or absence (–) of the specific 600 bp target product.

plus 5% nonfat dried milk at 25°C for 1 h, followed by rinsing three times in TBST for 5 min each. Next, the membrane was incubated with the primary antibody solution (diluted in TBST) for 1 h and rinsed three times, as above. Each membrane was incubated with the secondary antibody solution for 1 h, followed by rinsing three times, as described above. After soaking in freshly prepared fast blue/naphthol phosphate substrate solution for 20 min, background colour was removed by soaking in 1% sodium hypochlorite for 15 min, followed by rinsing in tap water. The membranes were placed between filter paper pads and either air-dried overnight or baked at 80°C for 30 min. The enzyme-substrate reaction produced a dark-blue insoluble precipitate on the membrane if the *X. albilineans* antigen was present.

Indirect ELISA

Pooled sap from individual stalks was spun down at 8160 ***g*** for 5 min. Indirect ELISA was performed, following the modified protocol of Comstock & Irey (1992). The primary and secondary antibodies were used as described for DIA. An absorbance reading above 0·05 A_{405nm} was considered positive, because background readings of negative controls ranged between 0·001 and 0·006.

Results and discussion

PCR specificity and sensitivity

Primers XAF1/XAR1 resulted in a 600-bp product from genomic DNA of *X. albilineans* (33915) (Fig. 1, lanes 15–20) and 18 other isolates of *X. albilineans* from various geographical locations (Table 1). Presence of the 600-bp product was confirmed by Southern hybridization (data not shown). None of the 10 isolates of other xanthomonads, *Escherichia coli* or *Pseudomonas syringae* gave visible bands after electrophoresis on agarose gels or were detected by Southern hybridization. Furthermore, no amplification product was produced

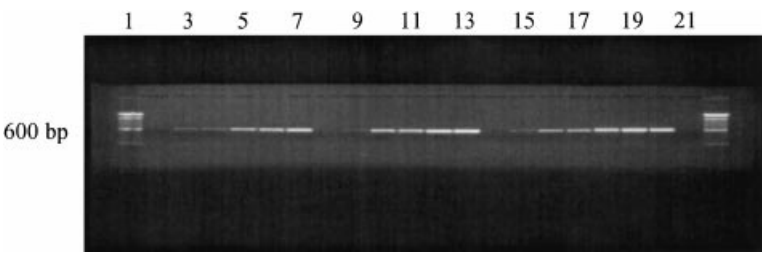

Figure 1 Sensitivity of classical PCR amplification in detecting viable cells of *Xanthomonas albilineans* in vascular bundle sap, in cell suspensions and genomic DNA. Lanes 1 and 22: 100 bp DNA ladder; 2–7: vascular bundle sap containing ≈ 1, 9, 110, 347, 2067 and 20 000 colony forming units (cfu) prior to amplification; 8–13: cell suspensions containing ≈ 6, 20, 110, 510, 2660 and 26 000 cfu prior to amplification; 14–20: genomic DNA of *X. albilineans* (FB648), containing 1 pg, 10 pg, 100 pg, 1 ng, 10 ng, 100 ng and 1 μg DNA; 21: negative control (FSDW).

from any of the five yellow xanthomonad-like bacteria commonly isolated from sugarcane stalks (Table 1) or from several other unknown bacteria isolated from sugarcane, soil or weed samples taken near infected sugarcane plants (data not shown). The 600-bp product of *X. albilineans* was consistently detected in samples of pure cultures and vascular bundle sap containing at least 2×10^4 cfu mL^{-1} of *X. albilineans*. Samples containing fewer than 2×10^4 cfu mL^{-1} (less than 20 cfu per PCR reaction) sometimes gave a very faint band on agarose gels after electrophoresis (Fig. 1 and Table 2). These sensitivity results agreed with results of others using a single set of external primers (Prosen *et al*., 1993; Slack *et al*., 1996). The specific amplification product was consistently detected using quantities of ≥ 10 pg genomic DNA of *X. albilineans* per reaction.

The DIA and ELISA techniques were the least sensitive, requiring populations of 10^6 mL^{-1} and 10^5 mL^{-1}, respectively, for positive detection, in agreement with previous results (Rott *et al*., 1988; Comstock & Irey, 1992). The four techniques (isolation, DIA, ELISA, and classical PCR) detected *X. albilineans* in 100% of the cases where symptom-bearing stalks from either young or mature plants were used. This level of detection is slightly higher than that reported earlier (Comstock & Irey, 1992; Davis *et al*., 1994). Differences among techniques were clearly evident when using tissues without symptoms, *X. albilineans* being detected by at least one of the techniques in 15·9% and 41·0% of these stalks from young and mature plants, respectively (Tables 3 and 4). The frequency of detection of *X. albilineans* varied with the technique. The ranking of the techniques from the least sensitive to the most sensitive for both young and mature symptom-free stalks was DIA, ELISA, classical PCR and isolation on m-XAM (Tables 3 and 4). For plants with unknown symptoms from burned commercial fields, the results were less varied. The pathogen was detected in 7·1, 8·9, 9·8 and 10·2% of the samples, by DIA, ELISA, classical PCR and isolation on m-XAM, respectively, agreeing favourably with results of potato tuber assays (Slack *et al*., 1996). Over all types of sugarcane samples, isolation on m-XAM agar medium consistently detected the highest percentage of infected plants and was the most sensitive assay technique. However, because isolation requires 8 days, the technique has limited usefulness.

To obtain consistent results using classical PCR, vascular bundle sap samples had to be either diluted or boiled for 10 min (Tables 3 and 4, Fig. 2). The boiling treatment was also necessary in obtaining consistent PCR results with leaf samples (data not presented). Using the boiling treatment, *X. albilineans* could be detected by classsical PCR in vascular bundle sap samples without extraction of DNA. The boiling probably destroyed PCR inhibitors. Other workers have observed such inhibitor problems interfering with PCR assays for seed samples (Prosen *et al*., 1993). The boiling treatment is preferred over diluting the sample since the PCR reactant volume is so small and samples containing low numbers of cells may be rendered undetectable by the dilution necessary to get rid of inhibitors. The NaOH treatment did not improve results and was not used further (Fig. 2).

Classical PCR assays were always positive for

Table 3 Detection of *Xanthanomas albilineans* by four different techniques (isolation, ELISA, DIA, and PCR assays) in 23 symptom-bearing and 132 symptom-free stalks of young sugarcane[ab]

Stalk sample	Isolation	ELISA	DIA	PCR	Number of stalks
With symptoms	+	+	+	+	23
Total sampled					23
Without symptoms	+	+	+	+	3
	–	–	–	–	111
	+	–	–	+	5
	+	–	–	–	11
	+	+	–	+	2
Total sampled					132
% detection by each technique					
With symptoms	100·0	100·0	100·0	100·0	
Without symptoms	15·9	3·8	2·3	7·6	

[a]Stalk samples of known disease state were taken from 4-month-old plants grown from *X. albilineans*-inoculated seedpieces at the Canal Point Sugarcane Field Station.

[b]Positive (+): for isolation consisted of typical *X. albilineans* colonies on m-XAM plates after 6 days' incubation; for ELISA a reading of 0·05 or greater at A_{405nm}, for DIA a visible blue dot; and for PCR the presence of a 600-bp target product. The combination of + and – indicates positive and negative detection patterns observed by the four techniques for the same sample.

Table 4 Detection of *Xanthanomas albilineans* by four different techniques (isolation, ELISA, DIA, and PCR assays) in 100 symptom-bearing and 100 symptom-free stalks of mature sugarcane[ab]

Stalk sample	Isolation	ELISA	DIA	PCR	Number of stalks
With symptoms	+	+	+	+	100
Total sampled					100
Without symptoms	+	+	+	+	8
	–	–	–	–	59
	+	–	–	+	11
	+	–	–	–	9
	+	+	–	+	13
Total sampled					100
% detection by each technique					
With symptoms	100·0	100·0	100·0	100·0	
Without symptoms	41·0	21·0	8·0	32·0	

[a]Stalk samples from 10-month-old plants of known disease state were collected from naturally infected cultivar development plots at the Canal Point Sugarcane Field Station. Five symptom-bearing and five symptom-free plants from 20 different clones were used.

[b]Positive (+): for isolation consisted of typical *X. albilineans* colonies on m-XAM plates after 6 days' incubation; for ELISA a reading of 0·05 or greater at A_{405nm}; for DIA a visible blue dot; and for PCR the presence of a 600-bp target product. The combination of + and – indicates positive and negative detection patterns observed by the four techniques for the same sample.

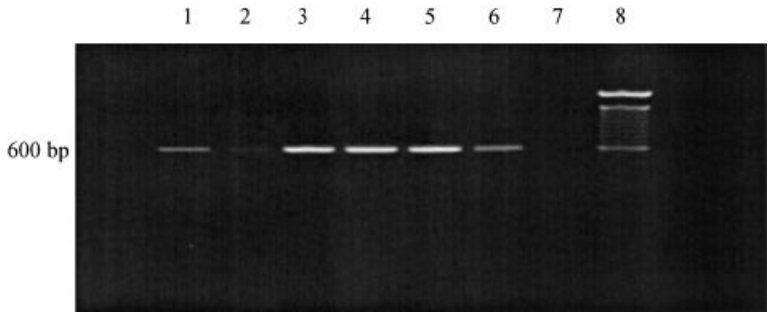

Figure 2 Effect of sample preparation of vascular bundle sap from stalks with leaf scald symptoms and of leaf exudates on PCR amplification. Lane 1: Boiled stalk sap (10 min); 2: washed stalk sap; 3: washed and boiled stalk sap (10 min); 4: washed and boiled leaf exudate from leaves with pencil-line symptoms; 5–7: alkaline (0·05 M NaOH) plus 0·1% pvp treated: stored 3, 14 and 30 days at 4°C, respectively; 8: 100 bp DNA ladder.

X. albilineans when leachates were from leaf tissues showing the pencil-line symptoms. This was predicted since *X. albilineans* is normally present in very large numbers and can be microscopically observed streaming from the vascular bundles of such leaves. In contrast, symptom-free leaf samples were always negative, indicating that the concentration of the pathogen was less than 1×10^4 cfu mL^{-1}.

In pure cultures and in a mixture with the yellow saprophytic bacterium, BIO-PCR was 100 times more sensitive than classical PCR (Table 5). Whereas BIO-PCR was positive for samples containing 2×10^2 cfu mL^{-1}, classical PCR failed to detect samples containing 3×10^3 cfu mL^{-1}. Neither classical PCR nor BIO-PCR were positive for undiluted samples containing very high numbers (1×10^5 cfu mL^{-1} or greater) of the target bacterium along with high numbers of other bacteria (Table 5). These results suggest that 1:100 dilutions should be included with all samples for BIO-PCR. The BIO-PCR results, detecting the slow growing *X. albilineans* in samples containing as few as 10 cfu per PCR reaction (286 cfu mL^{-1}) agree with the detection of 10 cfu of *Pseudomonas syringae* pv. *phaseolicola* per ml of bean seed washing reported by Schaad *et al.* (1995).

Of the 150 sugarcane stalks with unknown symptoms sampled from the commercial field after burning, 19·0, 10·0 and 16·7% were positive for *X. albilineans* by isolation on m-XAM, classical PCR, and BIO-PCR, respectively, suggesting that at least 10% of the stalks contained infections of at least 1×10^4 cfu mL^{-1}. Although there was little difference between plating on m-XAM and BIO-PCR, additional BIO-PCR positives might have been detected if larger volumes of stalk sap had been available (only 100 μl was used) and additional samples had been plated onto m-XAM. Also, as shown earlier (Table 5), negative results occurred in BIO-PCR

Table 5 Comparison of PCR and BIO-PCR techniques in detecting *Xanthomonas albilineans* alone and in combination with a commonly isolated yellow saprophytic bacterium from sugarcane stalks

	X. albilineans[a]		*X. albilineans* and Yellow Saprophyte		Yellow Saprophyte
Isolation CFU[b]	PCR	BIO-PCR[c]	PCR	BIO-PCR	BIO-PCR
ND	+	+	–	–[g]	–
133000[d]	+	+	–	–[g]	–
14000[d]	+	+	+	–[g]	–
4900[d]	+	+	+	+[g]	–
2900[e]	+	+	+	+[h]	–
620[e]	+	+	–	+[h]	–
120[e]	–	+	–	+[h]	ND
10[f]	–	+	–	–[h]	ND
0	–	–	–	–[h]	ND

ND: Not determined.

[a]Cells of *X. albilineans* from six-day-old cultures were suspended in sterile water ($A_{600nm}=0·1$) and a 10-fold dilution series (10^{-0}–10^{-8}) was performed. Thirty-five μl of each stepwise dilution was used for assays in 50 μl total volume. Suspensions of *X. albilineans and* yellow saprophyte were made by mixing an equal concentration of each organism and diluting as above.

[b]Number of colony-forming units (cfu) per PCR reaction for *X. albilineans*. The number also represents the approximate number of the yellow saprophyte. Counts were determined after 7 days at 28°C on m-XAM semiselective medium and multiplied by 28·6 to determine cfu mL^{-1} in the solution.

[c]BIO-PCR assays were performed on suspensions obtained by washing culture plates that had been incubated 4 days at 28°C.

[d]Pin-point colonies of *X. albilineans* visible after 48 h incubation.

[e]Pin-point colonies of *X. albilineans* visible after 72 h incubation.

[f]Pin-point colonies of *X. albilineans* visible after 96 h incubation.

[g]Colonies of yellow saprophyte bacterium visible after 24 h incubation.

[h]Colonies of yellow saprophyte bacterium visible after 48 h incubation.

when high numbers of yellow saprophytic bacteria were present. Assaying both zero and 1:100 dilutions of the plate washings might have resulted in additional positives for samples containing too few target bacteria or too many other bacteria, respectively. BIO-PCR still offers major advantages over agar plating: results do not need to be verified by pathogenicity tests and are available four days sooner, and fewer false negatives are obtained from samples containing high numbers of saprophytes. The major advantages of BIO-PCR over serology include a much greater sensitivity and the detection of only viable cells. These advantages are very important for a highly regulated quarantined pathogen such as *X. albilineans*. BIO-PCR and isolation on m-XAM were consistently more sensitive in detecting *X. albilineans* than DIA, ELISA or classical PCR. Classical PCR would consistently detect *X. albilineans* only in samples containing at least 2×10^4 cfu mL^{-1}. Because of the biological amplification, BIO-PCR assay is 100 times more sensitive. A major disadvantage of BIO-PCR over classical PCR is the additional three days required because of the slow growth of *X. albilineans*. Both isolation on m-XAM and BIO-PCR are recommended for assay of sugarcane stalks for *X. albilineans*. Primers other than XAF1/XAR1 that are selective for *X. albilineans* (Pan *et al*., 1997) could also be used for verification.

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